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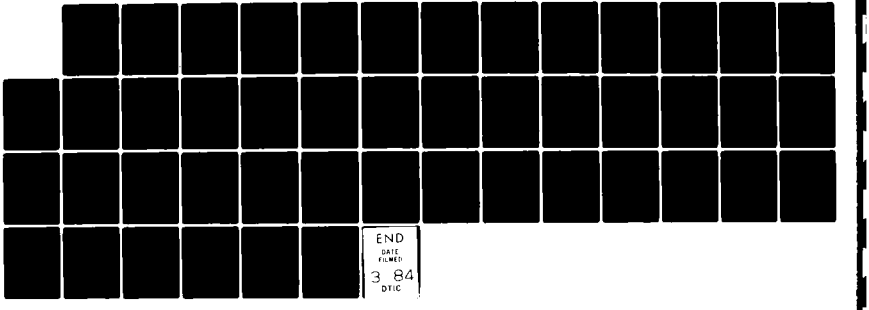

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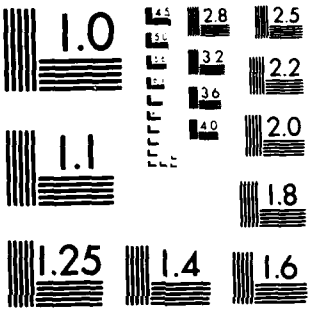

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report reviews progress made under contract DAMD17-79-C-9071. Preliminary studies were carried out using technetium-labelled RBCs in measuring red cell mass; the relationship between ATP levels and the viability of red cells was investigated; and several procedures for protein extraction from red cells were performed.

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BLOOD PRESERVATION STUDY

ANNUAL PROGRESS REPORT

E. BEUTLER

January 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

FORT DETRICK, FREDERICK, MARYLAND 21701

Contract No. DAMD17-79-C-9071

Scripps Clinic and Research Foundation

La Jolla, California 92037

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1. Study of blood stored in CPD and in modified CPD solutions containing adenine.

a. Background

CPD (citrate-phosphate-dextrose) has been routinely used as the standard medium for preservation of blood for many years. Red cells from blood which has been collected in CPD can only be stored for 21 days. After this, they are no longer sufficiently viable for transfusion. It has been clearly established that the addition of adenine to CPD preservative solution prolongs the length of time that red cells may be stored to 35 or 42 days (1). Moreover, higher concentrations of glucose than those present in CPD are required to sustain red cell concentrates for prolonged periods of time in adenine-containing media (2).

CPD-A1 contains 1.25 times the glucose content of CPD and sufficient adenine to provide a concentration of 0.25 mM in the blood-preservative mixture. Higher glucose concentrations were not incorporated into this preservative because of the existence of anecdotal reports that high glucose concentrations

increased the rate of fall of the pH of platelet concentrates. CPD-A1 was studied by a cooperative group (3) with the demonstration that red cells from whole blood stored for 35 days had viability of 80.53 ± 6.44% (x ± 1 S.D.) while red cell concentrates with a hematocrit averaging 75% had a mean viability of 71.38 ± 10.3% (x ± 1 S.D.). After these studies were concluded, it was discovered that the manufacturer of the blood bags had inadvertently prepared the bags from an obsolete plastic film, PL 130, rather than the new plastic formulation, PL 146, which had been intended. These studies were then repeated in PL 146, with results quite similar to those obtained in PL 130.

In both of these studies, it was observed that most or all of the glucose was exhausted from cells in some units packed at hematocrits of 75 ± 5% and stored for 35 days. There is reason for concern that under field conditions, where the storage temperature may sometimes rise to higher than 4° and where packing of the red cells, a parameter which is difficult to control, may frequently exceed 80%, that CPD-A1 may prove to be suboptimal for the storage of red blood cells. In the meantime, in vitro studies failed to show any deleterious effect of higher glucose concentrations on platelet function (4). For

this reason, two new preservatives, believed to represent improved formulations for storage of cell concentrates have been devised and manufactured by Fenwal Laboratories. These preservatives designated CPD-A2 and CPD-A3 contain respectively 1.75 and 2.00 times the amount of glucose present in CPD and sufficient adenine to provide a concentration of 0.5 mM in the blood-preservative mixture.

Data which have been accumulated over the past 15 years clearly indicate that ATP levels are a disappointing parameter with respect to predicting the viability of stored red cells (5-8). There is a great need to identify measurements which may prove to be more useful than ATP in predicting whether or not a given storage system provides red cells which will be viable on reinfusion. Moreover, there is controversy regarding the best means of measuring red cell viability in human subjects. Back-extrapolation of ^{51}Cr activity after infusion of labelled, stored erythrocytes can be utilized (5). This is the technique which we have been using for the past 15 years. Alternatively, some investigators, fearing that early loss of non-viable stored transfused cells may produce an overestimate of the viability of stored erythrocytes, have made an independent measurement of plasma volume to estimate the red cell

mass. This procedure subjects the recipient to an increased amount of radioactivity, and it must be recognized that estimates of red cell mass derived from the plasma volume are not accurate because of the variable ratio of total body hematocrit to venous hematocrit. The development of an alternative method of labelling red cells with ^{99m}Tc now provides us with an alternative to adequately test the accuracy of the back-extrapolation technique for measurement of red cell mass.

b. Progress in current period (January 1982 through December 1982).

Biochemical studies and studies of viability of stored cells.

In the 1981 contract year studies were initiated in which red cells were packed to a hematocrit of 80% and stored for 42 or 49 days in the standing or lying position. Biochemical studies were performed on a total of 21 units, 13 stored for 42 days, and 8 stored for 49 days. Viability studies were performed on all but two of these units in which there was a suspicion of bacteriologic contamination (not subsequently confirmed). These investigations revealed a difference in glucose consumption between the lying and standing position

when blood was stored for 42 days, but no difference could be ascertained after 49 days storage. Since these data were difficult to understand, we studied additional units of blood from the same donors on whose blood viability studies had been performed. Blood from donors whose blood had been stored. in the lying position were now stored upright and visa-versa. These findings confirmed our earlier findings of the effect of storage position on glucose concentrations, and have been incorporated into a manuscript entitled Storage of red cell concentrates in CPD-A2 for 42 and 49 days (Appendix I).

Preliminary studies of the use of the technetium labelling technique in measuring red cell mass have been undertaken. The following procedure was utilized:

An aliquot from a Technetium PYP reaction vial (Technetium ^{99m}Tc Pyrophosphate Kit, Mallinckrodt Diagnostics), containing 12 mg sodium pyrophosphate and 3.4 mg stannous chloride in 10 ml, was diluted 1 to 100 with sterile non-pyrogenic saline for injection. 0.25 ml of this dilution, containing 2.1 ;g of tin per ml, were added to the blood with gentle mixing. This provides an approximate concentration of 0.23 g tin per ml RBC (assuming a hematocrit of 45%). After 5 minutes at room temperature the blood was centrifuged and as much of the plasma as

possible removed. 15 Ci of ^{99m}Tc Technetium pertechnetate were added to the RBCs and the suspension mixed gently. After 15 min at room temperature the cells were washed with 45 ml of saline. The supernatant after centrifugation was removed and the cells resuspended in one volume of saline.

After the method was tried in rabbits, the red cell mass of a patient with polycythemia was estimated using a mixture of the patient's fresh cells labelled with ^{51}Cr and fresh cells labelled with ^{99m}Tc. In this patient the estimated red cell mass was 2537 ml using the technetium method and 2484 ml using the ^{51}Cr method, an agreement to within less than 0.1%. The ratio observed, 1.021, was remarkably close to that of 1.01 reported by Jones and Mollison (6). Within the past week blood was drawn from two donors to now perform the type of studies outlined in our contract proposal.

2. Studies of red cell membrane

a. Background

Although it has been known for many years that some relationship exists between the ATP levels of liquid-red cells and their viability, the relationship between these two parameters is a weak one (7,8), particularly when ATP levels are

maintained at relatively normal concentrations for prolonged storage periods by the addition, for example, of adenine and phosphate (9). Under these circumstances the Achilles heel of the stored erythrocyte is not the ATP level but some as-yet-unknown factor. It may well be that gradual denaturation of red cell membrane proteins plays a role in this loss of erythrocyte viability. The past few years have seen great progress in our understanding of the red cell membrane (10,11,12), and there are now numerous studies which indicate that various sites of red cell membrane proteins interact with one another. It is to investigate interaction between various membrane proteins that we have undertaken studies to determine whether membrane protein interaction can be measured in a simple, reproducible fashion.

b. Progress in current period (January 1982 through December 1982)

We have developed techniques for solubilizing over 95% of red cell membranes. Blood was defibrinated and filtered through cellulose-microcrystalline cellulose to remove white cells and platelets. After the cells were washed three times in 0.9 sodium chloride solution they were lysed in 25 volumes of ice-cold 10 mM Tris hydrochloride, pH 8.0. After centrifugation in the cold at 35,000 g for 15 min the sedimented stroma

were washed three times in the same buffer. They were then washed an additional two times in 5 mM sodium phosphate buffer, pH 8.0. Two extractions were then performed, a lower ionic strength extraction designed to remove the extrinsic membrane proteins and a detergent extraction to solubilize the intrinsic proteins. In addition, trials were performed with high ionic strength extractions using 0.5 mM sodium chloride solution. The preliminary results of extractions in such a system are shown in Table I.

Having found that 0.1 mM EDTA provided good extraction of the extrinsic proteins and that 0.1% Triton X-100 provided satisfactory extraction, presumably of the intrinsic proteins, combinations of these two types of extractions were studied sequentially. The results of such studies are shown in Table II. The amount of protein remaining in the pellet after such extractions varied between only 1.3 and 10.6%. The pellet, moreover, contained an assortment of normal membrane proteins as revealed by SDS gel PAGE, indicating that no single component was being left behind in the extraction procedure.

We have now begun to study the reassembly of the extracted proteins after the addition of calcium and magnesium, monitoring the formation of large molecular weight aggregates using a

Turner 111 fluorometer with a 760 primary and a neutral density secondary filter. Baseline readings were obtained on the combined low ionic strength and detergent extracts and the signal was followed after the addition of calcium and magnesium. Adding calcium alone to these extracts did not produce an increase in light scattering in a fluorometer. However, when both calcium and magnesium were added at final concentrations ranging from 0.2 to 1.0, considerable amount of increased light scattering was observed. Centrifugation of samples treated in this way revealed that most of the membrane proteins were sedimented in this procedure. However, some precipitates also formed in blank tubes in which no protein was present. These presumably represented magnesium or calcium phosphate salts.

Accordingly, the extraction procedure has been revised so that the washing of the stroma in phosphate buffer was eliminated. Under these circumstances an increase in the signal also occurs when calcium and magnesium chloride are added to concentrated stromal preparations but not to solutions lacking stromal proteins.

We have performed preliminary studies using such extracts concentrated to a final cuvette concentration of 1 mg per ml. An increase in the fluorometer signal presumably due to light

scattering from molecular complexes formed when both magnesium and calcium were added. The effect of various concentrations of calcium and magnesium have also been investigated and are being studied further at various levels of protein in the cuvette.

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Table I

Extraction Procedure	Temp	% Protein Extracted	% Protein in Pellet
Control 1:10 5mM NaH_2PO_4/Na_2HPO_4	4°	2.2	97.8
	37°	3.4	96.6
1:10 0.1mM EDTA pH 8.0	37°	30.1,28.9	69.9,71.1
1:10 0.1mM EGTA pH 8.0	37°	35.1,30.2	64.9,69.8
1:2 0.5M NaCl in 5mM NaH_2PO_4/Na_2HPO_4	4°	5.0	95.0
Control 1:10 5mM NaH_2PO_4/Na_2HPO_4	4°	0	100
1:5 0.1mM EDTA	37°	27.8	72.2
1:10 0.1mM EDTA	37°	25.6	74.4
1:50 0.1mM EDTA	37°	36.8	63.2
1:100 0.1mM EDTA	37°	39.6	60.4
1:250 0.1mM EDTA	37°	43.6	56.4
Control 1:10 5mM NaH_2PO_4/Na_2HPO_4	4°	0	100
1:6 0.1% Triton X	4°	36.8	63.2
1:6 1.0% Triton X	4°	43.8	56.2

All procedures were carried out at pH 8.0
Triton X-100 prepared in 5mM NaH_2PO_4/Na_2HPO_4

Table II

Extraction Procedure	Temp	% Protein Extracted	% Protein in Pellet
Cont 1:10 5mM NaH_2PO_4/Na_2HPO_4	4°	0	100
1:10 0.1mM EDTA	37°	26.9	73.1
1:5 0.1% Triton X	4°	34.1	65.9
Combination Extractions (Sequential)			
	Temp	% Protein Extracted	% Protein in Pellet
1:10 0.1mM EDTA	37°	28.8	
1:5 0.1% Triton X	4°	60.5	10.6
1:5 0.1% Triton X	4°	35.0	
1:10 0.1mM EDTA	37°	61.8	3.1
1:10 0.1mM EDTA	37°	31.7	
1:5 1.0% Triton X	4°	61.4	6.9
1:5 1.0% Triton X	4°	48.1	
1:10 0.1mM EDTA	37°	50.6	1.3
Control 1:10 5mM NaH_2PO_4/Na_2HPO_4	4°	0	
1:10 0.1mM EDTA	37°	27.9	
1:5 0.1% Triton X	4°	64.5	7.4
1:5 0.1% Triton X	4°	39.8	5.3
1:10 0.1mM EDTA	37°	54.9	
1:10 0.1mM EDTA	37°	24.8	
1:5 0.1% Triton X	4°	66.5	8.8
1:5 0.1% Triton X	4°	30.3	
1:10 0.1mM EDTA	37°	63.6	6.1
Control			
1:10 NaH_2PO_4/Na_2HPO_4 5mM	37°	0	
1:5 NaH_2PO_4/Na_2HPO_4 5mM	4°	0	100
1:10 0.1mM EDTA	37°	32.5	
1:5 0.1% Triton X	4°	48.5	19.1
1:5 0.1% Triton X	4°	28.4	
1:10 0.1mM EDTA	37°	55.6	16.0

All procedures were carried out at pH 8.0
Triton X-100 prepared in 5mM NaH_2PO_4/Na_2HPO_4

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Appendix I

STORAGE OF RED CELL CONCENTRATES IN CPD-A2 FOR FORTY-TWO AND FORTY-NINE DAYS

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This paper is dedicated to Prof. S.M. Rapaport on the occasion of his 70th birthday, in recognition of his seminal contributions to the study of red cell metabolism.

This work supported in part by contract DAMD 17-79-C-9071 from the U.S. Army and grant HL 25552 from the National Institutes of Health, Heart, Lung and Blood Institute.

ABSTRACT

CPD-A2 is a modified citrate-phosphate-dextrose (CPD) blood preservative with adenine, containing 1 1/2 times as much glucose as CPD. Units (450 ml) of blood from 21 normal donors were collected in CPD-A2 in plastic bags and held at room temperature for 8 hrs. An 80% red cell concentrate was prepared and this was stored for 42 or 49 days at 4°C with the containers either in a standing or in a lying position. Measurements of glucose consumption, red cell ATP and 2,3-DPG, and of plasma hemoglobin, pH, Na^+ and K^+ were performed on all samples. The size of the "fragile tail" of osmotically fragile red cells was estimated in 12 samples. The post-storage 24 hr viability of their own stored ^{51}Cr tagged red cells was documented in 19 of the volunteers. At least 4 months after the original donation, a second unit of blood was collected from 8 of the donors to make possible intra-donor comparison of the biochemical effects of storage position.

After 42 days but not after 49 days of storage, red cells in concentrates stored in the lying position had consumed more glucose and had a higher post-storage pH than did cells stored in the standing position.

The post-storage 24 hr viability of red cells stored for 42 days averaged 83.6%, with all units exceeding 70% viability. At 49 days the average viability was 69.1%. Although the average viability of cells stored in the lying position for 42 days was higher than that of concentrates stored standing, the difference was not statistically significant at the 5% level.

The plasma hemoglobin level showed a weak correlation with viability of stored cells. Red cell ATP levels were correlated with viability only at 42 days' and not at 49 days' storage.

Concentrates of red cell collected in CPD-A2 manifested fully satisfactory viability for 42 days. At 49 days storage the results of viability studies were borderline. High plasma hemoglobin values are observed at both 42 and 49 days' storage, and may limit the usefulness of red cell concentrates stored for prolonged periods of time.

Liquid storage of blood continues to be the mainstay of bloodbanking. With growing appreciation of the value of plasma components and platelets, there has been an increasing tendency to store red cells as concentrates, often with a hematocrit of 80% or higher.

In vitro studies demonstrated that the amount of glucose provided by CPD was inadequate to supply the metabolic needs of packed red cells for longer than 28 days of storage (1). Accordingly, the adenine-containing preservative CPD-A1 was designed to contain 1.25 times the amount of glucose contained in CPD. However, it was apparent that even at a 70% hematocrit the amount of glucose in CPD-A1 was borderline at 35 days' storage (2). CPD-A2 was therefore designed. It is a CPD-based preservative containing 1.75 times the amount of glucose as CPD and twice the adenine of CPD-A1. Whole blood and packed red cells stored in CPD-A2 were found to provide excellent viability after 35 days storage (3,4).

We have now investigated the viability of red cells packed to a hematocrit of 80% stored in this medium for 42 or for 49 days. At the same time we studied several other aspects of liquid storage. The position in which blood bags are stored has previously received only scant attention (3). In routine bloodbanking practice bags of blood are stored in the upright position, but in our laboratory the bags have generally been stored in the lying position. Although this might appear to be a trivial difference, the diffusion distances between overlying plasma and sedimented red cells are much greater when bags of cells are stored in a standing position than in the lying position.

Since it appeared in earlier studies (2) that blood stored in our laboratory (identified as laboratory A) had lower post-storage glucose levels, we wished to determine whether the position of storage had any influence either on glucose consumption or on post-storage viability.

We have recently demonstrated that when stored red cells are freed of lactate by equilibration with isotonic saline solution, the osmotic fragility of most of the red cells approaches normal (4). If 2,3-DPG is regenerated, restoring the osmolarity of cells, the osmotic fragility curve is normalized except for a "fragile tail" of cells which have a reduced surface/volume ratio. An additional purpose of the studies we report here was to determine whether the size of this cell population could be used to predict post-infusion viability.

MATERIALS AND METHODS

All studies were carried out after obtaining informed consent from normal volunteer subjects under a protocol approved by the Institutional Human Research Committee.

450 ml of blood was drawn into 63 ml of CPD-A2 in PL 146 polyvinyl chloride blood bags obtained from Fenwal Laboratories. The composition of CPD-A2 is as follows: 15.6 mM citric acid, 89.6 mM sodium citrate, 225 mM glucose, 18 mM monobasic sodium phosphate and 4 mM adenine. Baseline plasma glucose, hemoglobin, pH (4°C), sodium, and potassium estimations were performed on blood refluxed from the collecting bag immediately after collection using standard techniques (7,8). After

being held for 8 hrs at room temperature the blood was centrifuged and, based on the donor's hematocrit, a sufficient amount of plasma was expressed to give a hematocrit of about 80%. The units of blood were then stored in a 4°C blood bank refrigerator without mixing.

To further document the within-donor effect of storage position on glucose consumption second units of blood were donated by 4 of the donors whose red cells has been previously stored for 42 days and 4 donors whose red cells had previously been stored for 49 days. At least 128 days had elapsed after the original donation. In each of these studies, glucose consumption, red cell ATP levels, and plasma hemoglobin levels were measured on blood stored in the position opposite to that originally used.

The size of the "fragile tail" after storage was estimated using techniques which we recently described in detail (4). In the case of samples which have been stored for 42 days, osmotic fragility curves were performed by measuring ^{51}Cr release from the washed, labeled red cells just prior to infusion. In the case of samples stored for 49 days, one aliquot of the cells was equilibrated for an hour with an isotonic phosphate-buffered saline solution at room temperature; another aliquot was equilibrated for one hour with an isotonic buffered saline solution containing 10 mM pyruvate, 10 mM phosphate and 10 mM inosine to regenerate red cell 2,3-DPG to near-normal levels. In each case, the osmotic fragility was determined by measuring hemoglobin release. The fragile tail was defined as the proportion of erythrocytes hemolyzing at a sodium chloride equivalent of either 0.55% or 0.50%, whichever

produced the first measurable hemolysis in fresh cells from the same donor.

In most instances the viability of the stored red cells was studied after reinfusion. In the case of these samples, 10 ml was taken from the bag 3-5 days before the end of the storage period for culture. On the 42nd or 49th day after storage approximately 10 ml of the packed cells were removed for viability studies. Four to 8 μCi of ^{51}Cr-sodium chromate were added to each sample and after 20 minutes at room temperature 40 ml of sterile, preservative-free isotonic saline were added, the sample was mixed and centrifuged at 1000 g for 10 minutes. The supernatant was removed and the cells were mixed once again in 40 ml of the isotonic saline. The supernatant after recentrifugation was removed and a 50% suspension of the red cells in isotonic saline was injected rapidly into an antecubital vein of the original donor. Blood samples were taken from the opposite arm at 5, 10, 15 and 20 minutes and after 24 hrs. The T_0 radioactivity was determined by back-extrapolation of radioactivity of whole blood samples drawn at 5 to 20 minutes corrected, in most cases, for minor changes in the hematocrit. The 24 hr viability was calculated by dividing the counts per minute (cpm) in the 24 hr sample by the cpm in the back-extrapolated T_0 sample. Standard methods of statistical analysis were used in curve fitting and in calculating correlation coefficients and Student's t values. All standard deviations are presented as the best estimate of the population variance.

RESULTS

Effect of Storage Position

a. 42 days' storage (Table I)

Glucose consumption of cells stored in a lying position averaged 751 ± 50 nMoles per ml RBC per day. Cells stored in the standing position consumed 662 ± 29 nMoles of glucose per ml RBC per day. With t = 4, the difference in glucose consumption was significant at the 0.01 level.

Hemolysis was significantly greater in the samples stored in the standing position, with a plasma hemoglobin of 2378 ± 513 mg/dl (mean ± S.D.), compared with a value of only 910 ± 234 mg/dl in the concentrates stored in a lying position. Although this difference is statistically significant ($t=7$; $p<0.01$) it seems likely that the samples stored in the standing position had a spuriously high plasma hemoglobin content: the 4 samples stored for 49 days actually manifested lower plasma hemoglobin levels than were observed in the samples which had stood for 42 days (see below).

A slight difference in post-storage pH was also documented. The pH of the concentrates stored for 42 days in a lying position was 6.851 ± 0.061 while the pH of those stored in a standing position was 6.793 ± 0.026 (mean ± 1 S.D.). This difference was significant ($t=2.4$; $p=0.05$). No other significant differences in the biochemical parameters measured were observed.

At 42 days' storage the mean 24 hr viability of the samples stored in the lying position was 85.3 % ± 6.9 % (mean ± 1 S.D.) while the viability of samples stored in the standing position averaged 80.5 % ± 4.1 %. With a t value of 1.4 this difference was not significant at the 0.05 level.

b. 49 days' storage (Table II)

After 49 days' storage, there appeared to be no significant difference between glucose consumption of samples stored in the lying or standing positions. Concentrates stored in the lying position consumed 636 ± 63 nMoles of glucose per ml of RBC per day, while cells stored in the standing position consumed 658 ± 103 nMoles of glucose per ml RBC per day.

In contrast to our observations at 42 days' storage, there was no increased hemolysis of samples stored for 49 days in the standing position. Indeed, hemolysis was somewhat less in these samples, 1435 ± 753 mg/dl compared with 2378 ± 513 mg/dl for the samples stored in the lying position. This finding casts doubt on the validity of the opposite difference observed for 42 days' storage (see above). No other significant differences in the biochemical parameters measured were observed.

At 49 days' storage the percent viability of cells stored in the lying position had declined to 68.78% ± 4.52%, while cells stored in the standing position had an almost identical viability of 69.4 ± 6.52%.

c. Paired comparisons

Tables III and IV compare the intradonor effect of storage in the lying position and standing position at 42 and 49 days. These studies confirmed the fact that storage position influenced glucose consumption after 42 but not after 49 days (Table III). Although hemolysis appeared to be slightly greater in the standing than in the lying position, the difference was not statistically significant, either at 42 or 49 days (Table IV). There was no significant difference between units stored in the lying and standing position with respect to ATP levels.

Predictors of Post-storage Viability

In evaluating predictors of red cell viability the results observed in samples stored in the lying and standing positions have been pooled.

a. Post-storage Red cell ATP levels

A significant correlation ($r = 0.85$; $t = 4$) was found between the 24 hr viability of red cells stored for 42 days and the post-storage ATP levels. However, as shown in Figure 1 a large change in ATP levels produced only a very minor alteration in viability. After 49 days' storage the relationship between 24 hr viability and post-storage ATP levels was not significant ($r = 0.07$; $t = 0.17$).

b. Plasma hemoglobin levels

Analysis of regression between plasma hemoglobin levels and viability after 42 days' storage provided a correlation coefficient of -0.489 with a t value of 1.7; at 49 days the correlation coefficient was -0.694 with a t value of 3.36. Thus, a weak but statistically significant correlation between plasma hemoglobin levels and viability could be demonstrated at 49 days' storage.

c. The osmotically fragile tail of red cells

When considered at either 42 or 49 days' storage, no significant correlation was observed between the size of the "fragile tail" of the stored cells measured either after removal of lactate alone (Fig. 2), on the one hand, or after removal of lactate and restoration of intracellular osmolarity by regeneration of 2,3-DPG (Fig. 3). A statistically significant correlation was observed when data from 42 and 49 days' storage was pooled and the size of the "fragile tail" after removal of lactate compared with viability. However, the results of such pooled analysis are considered of doubtful importance since both the size of the "fragile tail" and the decrease in viability are functions of time.

DISCUSSION

Remarkably good post-storage 24 hr viability was observed in red cells concentrates with a hematocrit of 80% when blood was collected in CPD-A2. Even after 49 days of storage, three of the eight samples manifested viability exceeding 70%, and the average viability was nearly 70%. All samples contained glucose at the end of storage. Thus this new medium apparently contains sufficient glucose and adenine to maintain metabolism of red cells for 6 or 7 weeks, even when they are held at room temperature for 8 hrs prior to refrigeration.

Although viability was satisfactory, the extent of in vitro hemolysis was high, reaching 2.5 g/dl in some samples, even after storage for only 42 days. Such values cannot be compared directly with plasma hemoglobin levels encountered in stored whole blood, since the plasma volume in 80% red cell concentrates is only about 1/6 of that in whole blood. Representing lysis of less than 2% of the stored cells, the amount of hemolysis is nonetheless considerably greater than that which occurs in whole blood stored for the same length of time. Little is known about the effect of transfusing this quantity of hemolysed red cells, but it may be that this level of hemolysis will be considered to be unacceptably high and that washing of the red cells prior to transfusion will be required.

Red cell ATP levels have been commonly used as a predictor of the post-storage viability, even in very recent publications (9). However, several studies have demonstrated that the relationship between

red cell ATP concentration and viability is a very weak one (10-12). This seems to be particularly the case after prolonged periods of storage, when other changes such as those occurring in the red cell membrane may become the limiting factors that determine whether the red cell will survive in the circulation. The present studies demonstrated once again that ATP levels are not a good predictor of red cell viability, particularly after 49 days' storage. Of course if the correlation between ATP and viability for 49 and 42 days were pooled, a strong relationship would appear to emerge. However, this type of comparison must be considered to be invalid. A great many biochemical changes in stored blood are time-dependent. Since post-storage viability is also time-dependent, it will correlate well with any other time-dependent parameter. Included would be such possibly related parameters such as ATP level and in vitro hemolysis, but also totally unrelated factors such as plasma lactate, plasma pyruvate, plasma Factor VIII level, and the number of viable leukocytes in the unit.

The osmotic fragility of stored red cells has long been known to be strikingly increased (13). The extent of this increase does not correlate with post-storage viability (14), and thus measurement of osmotic fragility has not proven to be useful in predicting viability of stored erythrocytes. We recently discovered that the strikingly increased osmotic fragility of stored red cells is largely due to changes in internal osmolarity. The principal factor giving rise to this change is the accumulation of lactate within erythrocytes. Since the membrane is only sparingly permeable to this solute (15), rapidly diluting stored

red cells in salt solutions results in a rapid influx of water because of the osmotic effect of the lactate (4). However, equilibrating the red cells at room temperature with a large volume of an isotonic sodium chloride solution for an hour removes the excess lactate from the erythrocyte (4). The second cause of the increased internal osmolarity of stored erythrocytes is the presumed replacement of the highly charged 2,3-DPG polyanion by Cl^-. When stored cells are depleted of lactate and incubated with inosine, phosphate, and pyruvate to restore 2,3-DPG levels, the osmotic resistance of the major portion of the stored cells becomes entirely normal (4). A sub-population of cells form a "fragile tail" in the osmotic fragility curve; these cells are preferentially removed after the stored cells are re-infused (4). It seemed possible that the size of this fragile tail of cells which had presumably lost membrane during storage might prove to be a good predictor of the percentage of cells which were viable upon post-storage re-infusion. However, in the present investigations little correlation was found between the size of this sub-population of red cells and the overall viability of stored erythrocytes.

Under ordinary blood banking circumstances, storage of red cells for 42 or 49 days may not be necessary or desirable. However, in certain places and under some conditions prolonged liquid storage may be important in maintaining an adequate blood supply. CPD-A2 would seem to be a very promising preservative medium for such purposes. The extent of hemolysis observed after prolonged storage represents an undesirable feature; washing of the cells prior to transfusion may be required.

Possibly the use of additives such as ethanol (16) or mannitol (17) which we have earlier shown to retard *in vitro* hemolysis, or the introduction of new plastic films in the manufacture of blood bags may prove to be helpful in this regard.

Figure 1. The relationship between post-storage ATP and 24 hr viability at the end of storage.

Figure 2. The relationship between the percentage of cells lysed in the osmotically fragile tail and the 24 hr viability after 42 or 49 days' storage. The size of the fragile tail was determined after removal of lactate as described in the text.

Figure 3. The relationship between the percentage of cells lysed in the osmotically fragile tail and the 24 hr viability after 49 days' storage. The size of the fragile tail was determined after removal of lactate and regeneration of 2,3-DPG to near-normal levels as described in the text.

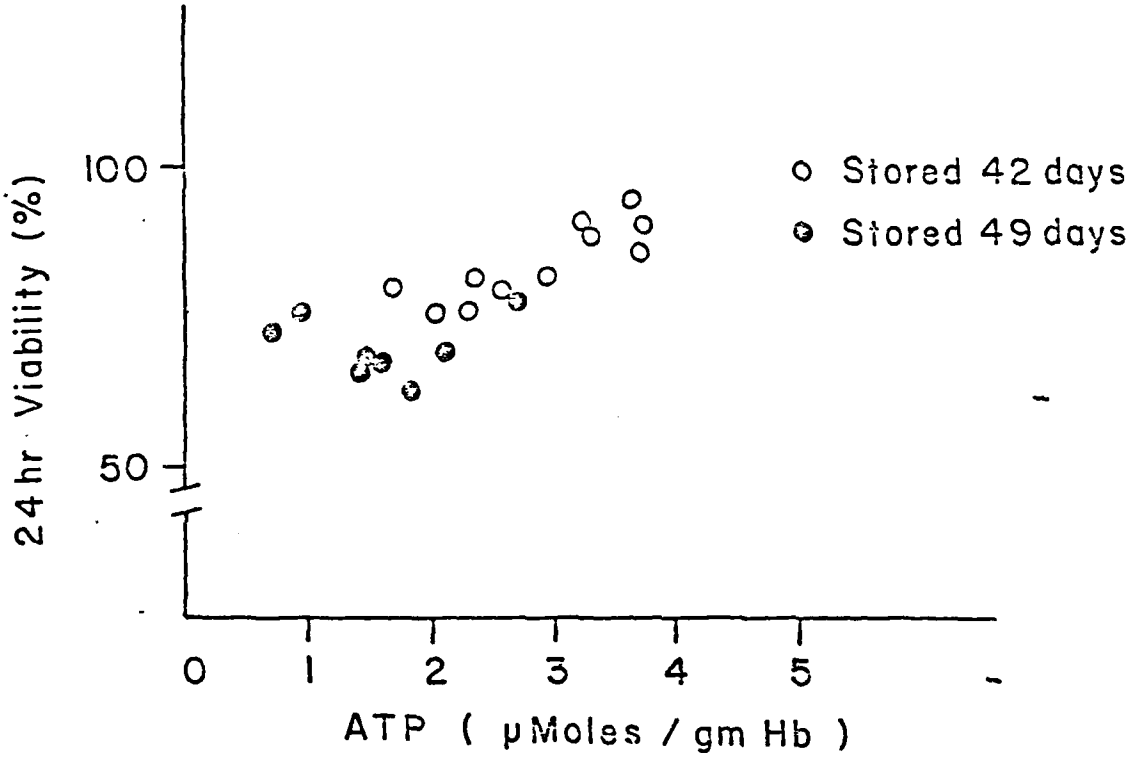
24 hr Viability (%)
100
50
0
1
2
3
4
5
ATP (µMoles / gm Hb)
Stored 42 days
Stored 49 days

FIGURE 1

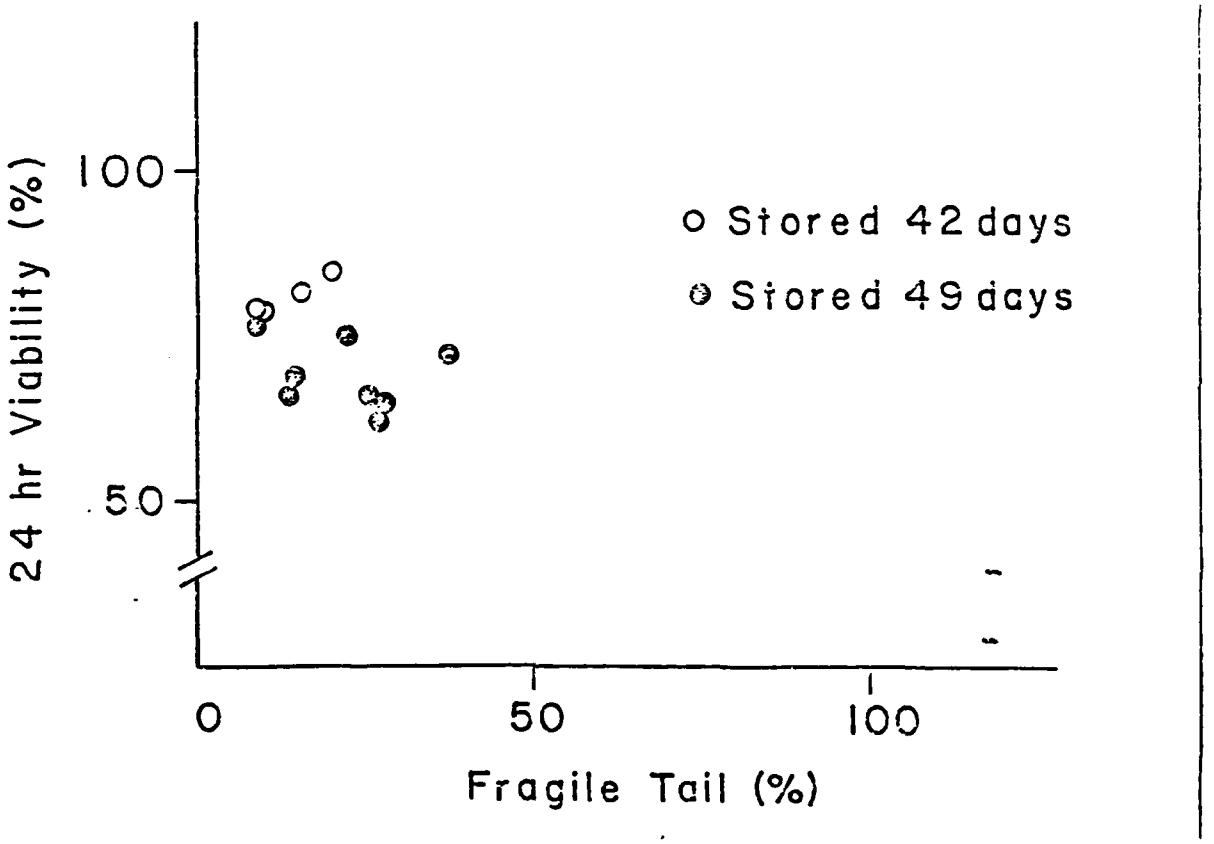
24 hr Viability (%)
100
50
0
50
100
Fragile Tail (%)
Stored 42 days
Stored 49 days

FIGURE 2

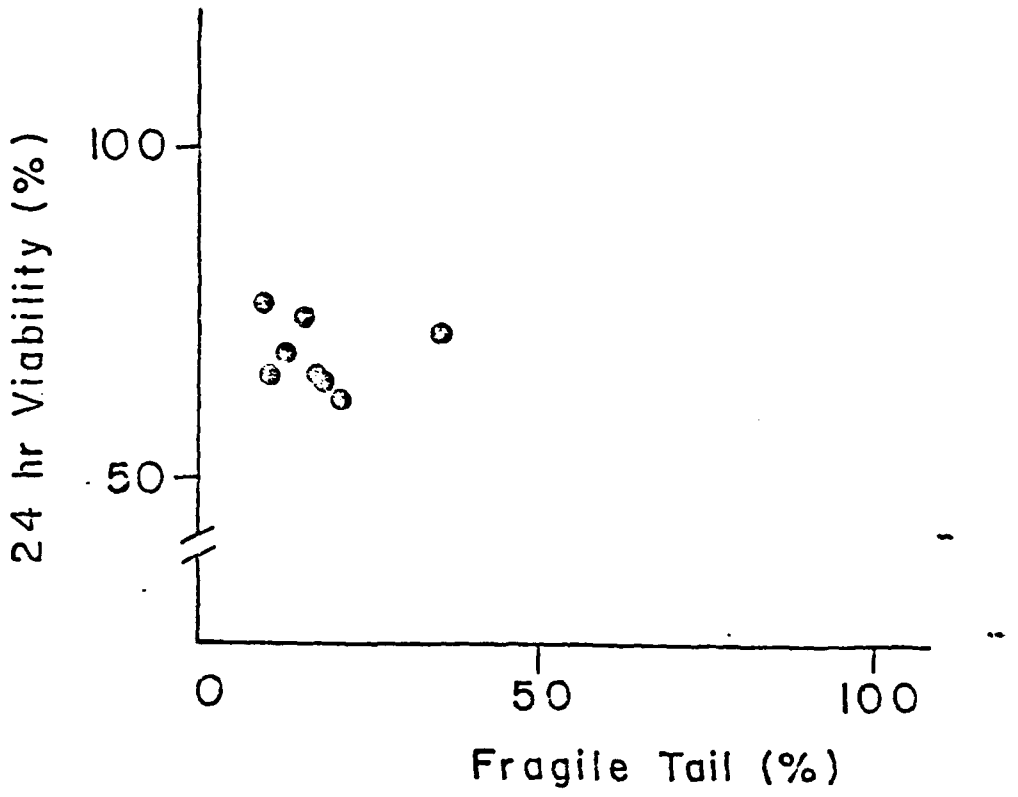

FIGURE 3

Table I. Results of 42 day storage of red cell concentrates in CPD-A2

Donor		MB	CC*	WC*	JS	MG	LP	DR	PG	AM	RP*	PN	KL*	TB
Storage Position (L = Lying, S = Standing)		L	L	L	L	L	L	L	L	L	S	S	S	S
	Day													
24 hr Viability (%)	42	93.6	90.1	81.4	90.4	87.9	N.D.	N.D.	78.9	75.1	85.4	79.2	81.6	75.6
Hematocrit (%)	0	35	39	35	39	36	34	39	29	33.5	37	34	41	43
	42	80	78	80	81	80	84.5	80.3	80	81	85	80	81	80.8
Glucose (mg %)	0	549	589	587	599	590	553	517	531	598	526	534	566	564
	42	97	137	148	127	115	95	76	124	79	127	129	146	153
ATP (μMoles/gm Hb)	0	5.03	4.05	3.80	4.60	4.20	4.71	4.48	3.87	4.16	4.39	3.86	4.49	4.40
	42	3.61	3.71	2.38	3.22	3.27	3.56	3.62	1.68	2.07	3.71	2.55	2.96	2.30
2,3 DPG (μMoles/gm Hb)	0	14.9	11.2	12.8	14.6	11.6	12.5	14.3	11.1	10.0	13.0	13.7	10.2	14.0
	42	0.3	0.2	0.1	0.3	0.3	0.1	0.1	0.2	0.1	0.5	0.2	0.2	0.1
Plasma Hb (mg %)	0	14	15	17	19	10	12	26	10	7	11	10	20	21
	42	420	268	720	860	910	2263	264	613	1875	2775	2263	1750	1568
pH (4°C)	0	7.57	7.61	7.57	7.63	7.58	7.61	7.51	7.43	7.50	7.74	7.53	7.55	7.62
	42	6.81	6.92	6.87	6.87	6.92	6.91	6.78	6.76	6.82	6.77	6.79	6.78	6.83
Na^+ (mEq/L)	0	169	174	170	171	174	181	171	166	170	182	172	176	174
	42	94	109	97	87	92	77	83	76	80	75	80	81	82
K^+ (mEq/L)	0	3.3	3.1	3.4	3.3	3.3	3.9.	3.6	3.2	3.3	3.3	3.6	3.3	3.3
	42	97	83	94	100	92	117	104	102	98	114	98	102	102
Glucose Consumption (nMoles/ml RBC/day)	42	747	766	726	771	786	717	726	673	848	620	669	687	673

*Studies of glucose consumption and plasma Hb were repeated after >128 days in the opposite storage position. Results are given in Tables III and IV.

Table II: Results of 49 days' storage of red cell concentrates in CPD-A2.

Donor		KC*	LH*	KH	JW	LF*	YM*	KH	ML
Storage Position		L	L	L	L	S	S	S	S
L = Lying S = Standing									
	Day								
24 hr Viability (%)	49	75.1	66.0	65.1	68.9	72.2	66.2	62.2	77.0
Hematocrit (%)	0	33.9	37.5	32.0	39.0	39.7	33.8	32.0	36.4
	49	83.3	80.5	80.4	82.5	81.3	80.3	79.8	80.9
Glucose (mg %)	0	548.5	538.4	555.3	555.9	619.9	526.5	555.8	569.1
	49	60.0	94.6	163.5	47.6	40.1	99.4	146.9	112.8
ATP (μMoles/gm Hb)	0	3.54	3.85	3.33	3.89	4.28	4.45	4.47	4.78
	49	0.95	1.55	1.45	2.11	0.76	1.47	1.83	2.69
2,3-DPG(μMoles/gmHb)	0	17.05	13.28	10.17	14.28	17.61	11.86	12.52	13.90
	49	0.10	0.10	0.08	0.12	0.10	0.05	0.09	0.07
Plasma Hb (mg %)	0	11.0	5.8	10.8	5.8	14.4	7.8	10.8	7.8
	49	1624	2700	2490	2700	1248	1192	2520	780
pH (4°C)	0	---	---	---	---	---	---	---	---
	49	6.76	---	6.9	6.79	6.66	6.70	6.76	6.79
Na^+ (mEq/L)	0	164	166	166	167	164	169	165	168
	49	78	74	74	68	77	80	70	80
K^+ (mEq/L)	0	2.8	3.3	3.3	3.1	2.8	3.1.	3.4	3.3
	49	115	111	111	128	117	112	113	113
Glucose consumption (nMoles/ml RBC/day))	49	666	625	553	698	808	603	580	639

*Studies of glucose consumption and plasma Hb were repeated after > 128 days in the opposite storage position. Results are given in Tables III and IV.

Table III. Glucose Consumption (nMoles/ml RBC/day) of paired red cell concentrates obtained from 8 donors and stored in the lying and standing position.

A. 42 Days Storage

Donor	Lying (L)	Standing (S)	(L-S)
CC	766*	685**	+ 81
WC	726*	628**	+ 98
RP	743**	620*	+123
KL	729**	687*	+ 42
	mean ± 1 S.E.		+ 86 ± 17

B. 49 Days Storage

KC	666*	695**	- 29
LH	625*	672**	- 47
LF	731**	808*	- 77
YM	737**	603*	+134
	mean ± 1 S.E.		- 5 ± 47

*First donation
**Second donation (>128 days after 1st)

Table IV. Plasma Hb(mg%) of paired red cell concentrates obtained from 8 donors and stored in the lying and standing position.

A. 42 Days Storage

Donor	Lying (L)	Standing (S)	(L-S)
CC	268*	570**	- 302
WC	720*	675**	+ 45
RP	850**	2775*	-1925
KL	1065**	1750*	- 685
	mean ± 1 S.E.		- 717 ± 430

B. 49 Days Storage

KC	1624*	2700**	-1076
LH	2700*	4170**	-1470
LF	2800**	1248*	+1552
YM	1950**	1192*	+ 758
	mean ± 1 S.E.		- 59 ± 724

*First donation
**Second donation (>128 days after 1st)

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